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Solution conformation of biantennary complex type oligosaccharides

Determination of major conformers about the glycosidic linkages

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Using data obtained from both one-dimensional and two-dimensional Nuclear Overhauser effect measurements, we have extended our original observations [FEBS Lett. (1983) 150, 503–506] upon the preferred conformer of the complex-type oligosaccharide unit derived from human serum transferrin. We propose that the Galβ1–4GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc unit has continuous secondary structure, and that the α(1–6) antenna extends from this structure with no single preferred orientation. Within the α(1–6) antenna, however, a segment of secondary structure (Galβ1–4GlcNAcβ1–2Manα1–) exists with a preferred conformation which is probably identical to that found in the α(1–3) antenna.

Oligosaccharide conformation *Nuclear Overhauser effect* *Human serum transferrin* *Two-dimensional NMR spectroscopy*

1. INTRODUCTION

The solution conformations of glycoprotein oligosaccharides and related sugars have been the subject of several studies [1–5]. In particular, we have demonstrated that the reduced asialo-biantennary complex type *N*-linked oligosaccharide isolated from human serum transferrin (HST) by hydrazinolysis contains regions of secondary structure [1]. Our initial study utilised one-dimensional (1-D) quantitative nuclear Overhauser effect (NOE) measurements. These studies were limited to measurements upon the resolvable resonances from the anomeric and H2 protons of the constituent monosaccharide residues. We have recently obtained new resonance assignments within the unresolved envelope of skeleton protons [6] which potentially allows for more structural information to be obtained. However, in crowded spectral regions, the measurement of NOE's using 1-D methods cannot provide unambiguous structural information, since the irradiating r.f. field has finite selectivity. To overcome this problem, we have used two-dimensional (2-D) nuclear Overhauser effect measurements [7] to probe those resonances within the unresolved envelope. The combination of our earlier quantitative 1-D NOE measurements [1] combined with semi-quantitative, 2-D NOE measurements are used here to obtain new structural information on the oligosaccharide chains derived from human serum transferrin.

2. MATERIALS AND METHODS

Human serum transferrin (HST) was obtained from Sigma. The complex-type biantennary oligosaccharide was released from HST by hydrazinolysis [8] and samples for NMR studies were prepared as in [6]. Unlike the previous study [6], however, investigations were performed upon the unreduced, fully sialylated chains from HST (fig.1).

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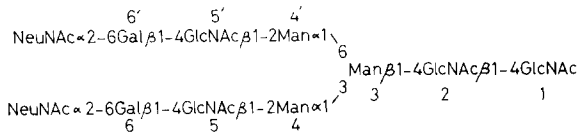

Fig.1. Structure of the disialylated oligosaccharide derived from HST used here. The numbering system is that referred to in the text.

One-dimensional nuclear Overhauser effect measurements were performed at 300 MHz as in [1]. Two-dimensional nuclear Overhauser effect measurements [7] were performed at 470 MHz at a probe temperature of 25°C, using the following pulse sequence:

$$90_\phi - t_1 - 90_\phi - \tau_m - 90_\phi - t_2$$

The phases of the first two pulses were cycled in 90° steps to cancel magnetisation arising from double quantum coherence. In addition, the phases of the first and third pulses were cycled in 90° steps to cancel both axial magnetisation and transverse interference arising from relaxation during the mixing period, τ_m. Finally, the phases of the second and third pulses were cycled in 90° steps to achieve quadrature phase detection in ω_1. In total, 64 transients (sweep width ± 1200 Hz, 2048 data points) were collected for each of 256 increments in t_1 (416 μs). Prior to Fourier transformation, time domain signals were weighted with phase-shifted sine-bell functions. After Fourier transformation, the 2-D data matrix was symmetrised [9] and displayed in absolute value mode. The contribution to crosspeaks in the 2-D spectrum from zero-quantum coherence remained unsuppressed in this study. We therefore restrict our NOE analyses to resonances which are essentially uncoupled.

Two-dimensional ^{1}H homonuclear correlated (COSY) experiments were performed essentially as in [6] but with fixed delays τ = 200 ms in both t_1 and t_2 periods, to emphasise crosspeaks arising from long-range couplings (J < 1 Hz) [10].

3. RESULTS AND DISCUSSION

The two-dimensional ^{1}H-^{1}H NOE spectrum of the unreduced, disialylated oligosaccharide derived from HST is shown in the form of a contour plot (fig.2). The conventional 1-D spectrum can be

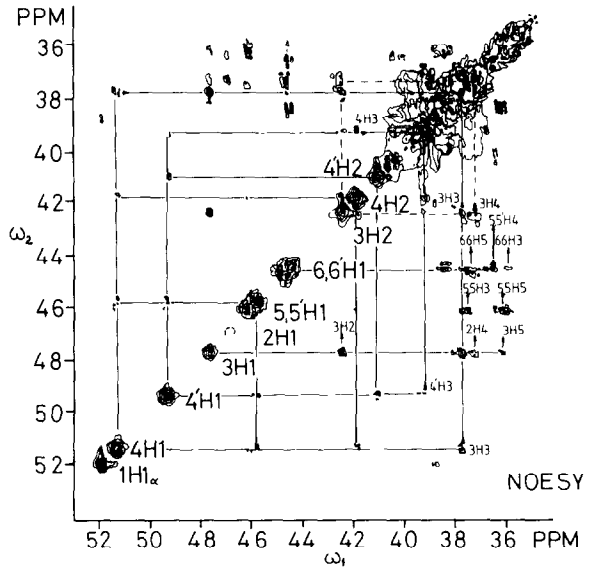

Fig.2. Two-dimensional NOE contour plot (3.5–5.2 ppm) of the reduced, disialylated oligosaccharide derived from HST. The region at ω_2 ~4.7 ppm, corresponding to the strong HOD signal, has been removed. The strong peaks on the diagonal correspond to the normal 1-D ^{1}H-NMR spectrum of the oligosaccharide. Crosspeaks showing proton–proton through-space connectivities appear symmetrically with respect to the diagonal. Several of these have been connected with solid lines as shown. The dashed line shows crosspeaks resulting from magnetisation transfer between the tightly coupled H3 and H4 protons of Man 3.

thought of as lying along the main diagonal from the lower left-hand corner to the upper right-hand corner of the contour plot. The off-diagonal peaks (crosspeaks) correlate resonances corresponding to protons which are through-space coupled by mutual dipole–dipole relaxation. Note that both inter- and intra-residue through space couplings can be observed in fig.2. The former are useful in the determination of the solution conformation of the molecule (through-space connectivities), whilst the latter are used to obtain new resonance assignments and to conform previous tentative assignments made on the basis of COSY [6]. The new resonance assignments obtained in this study are shown in table 1.

The NOE crosspeaks from the resolved protons in fig.2 (those labelled on diagonal) describe semi-quantitative connectivities. For example, a cross-

Table 1

Proton resonance assignments in the fully sialylated, unreduced oligosaccharide derived from HST by hydrazinolysis

Residue	2	3	4	4′	5,5′	6.6′
H1	4.64	4.76	5.13	4.94	4.60	4.44
H2	3.76	4.25	4.19	4.11	3.73	3.53
H3	3.73	3.77	3.89	3.87	3.76	3.65
H4		3.71[a]	3.58	3.60	3.70[a]	3.91
H5		3.62[a]	3.50[ab]		3.62[a]	3.73[a]

[a] Assignments obtained here
[b] Tentatively assigned at 3.79 ppm in [1]

section through ω_1 at ω_2 = 4.25 ppm is shown in fig.3. This clearly shows NOE's from Man 3H2 (diagonal) to Man 4H1, Man 3H1, and Man 3H3. In addition, three further crosspeaks are observed at 3.92, 3.71 and 3.61 ppm. From our previous studies [6] it is clear that the multiplet at ~3.71 ppm corresponds to Man 3H4, and the crosspeak at this position arises due to magnetisation transfer from Man 3H3, to which Man 3H4 is tightly coupled. This effect has been observed in similar compounds in conventional 1-D NOE studies [4]. The multiplet at ~3.61 ppm correlates well with our previous (tentative) assignment to Man 3H5. A crosspeak is expected at this position by virtue of the 3 spin-effect from Man 3H2 via both Man 3H3 and Man 3H1. Due to the absence of a firm assignment we are not yet able to interpret the crosspeak at 3.92 ppm.

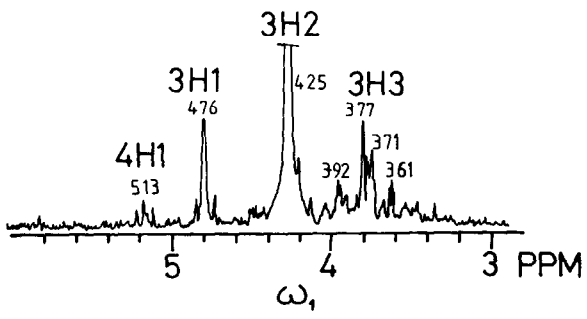

Fig.3. A cross-section along the ω_1 dimension of fig.2 at ω_2 = 4.25 ppm, showing the NOE connectivities from Man 3H2.

Using a combination of nuclear Overhauser effect measurements, coupling constant analysis and energy calculations, the authors in [4] have reported a conformation for the Manα1–3Manβ1– linkage in biantennary complex-type oligosaccharides which differs from the conformation proposed by us [1]. In particular, these authors have had to assume the existence of a large inter-residue NOE from Man 3H2 to Man 4H5 in order for their conformation to be compatible with their NOE data. The NOE observed from Man 3H2 to Man 4H1 [1] is then proposed [4] to occur from the 3 spin effect via Man 3H3, rather than a direct mechanism. The inability of these authors to observe directly the inter-residue NOE from Man 3H2 to Man 4H5 was thought to arise from the overlap of the resonances of Man 3H3 and Man 4H5, both of which should experience NOE's on saturation of Man 3H2 in their proposed conformation. Therefore, critical to their analysis is the Man 4H5 resonance assignment. Our investigations show that there is neither an overlap of the Man 4H5 resonance with the Man 3H3 resonance, nor an inter-residue NOE between Man 3H2-Man 4H5, thus making their proposed conformation inconsistent. We summarise our findings as follows: Firstly, the 1-D NOE difference spectra obtained upon irradiation of Man 4H1 and Man 3H2 appear to be identical with respect to the multiplicity of the resonances at 3.77 ppm (fig.4a,b). In both cases magnetisation transfer can be observed from Man 3H3 to Man 3H4 resulting in the observed multiplet pattern. An underlying NOE from Man 3H2 to Man 4H5 as

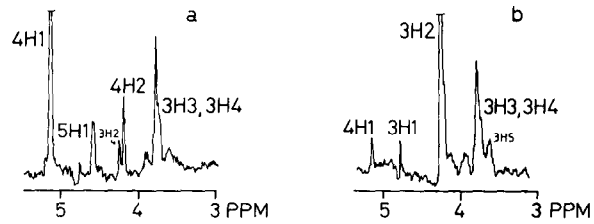

Fig.4. (a) One-dimensional NOE difference spectrum (300 MHz) obtained upon irradiation of the resonance corresponding to Man 4H1 (5.13 ppm). The strong HOD signal has been removed. (b) One-dimensional NOE difference spectrum (300 MHz) obtained upon irradiation of the resonance corresponding to Man 3H2 (4.25 ppm). The strong HOD signal has been removed.

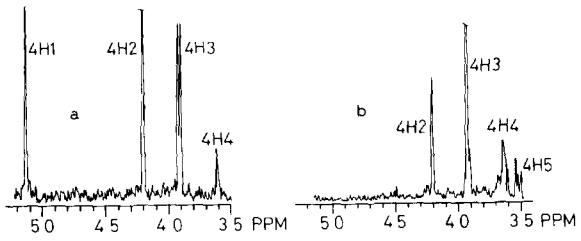

Fig.5. (a) A cross-section along ω_1 at ω_2 = 4.19 ppm of the COSY spectrum of the oligosaccharides derived from HST, showing through bond connectivities from Man 4H2. (b) A similar cross-section at ω_2 = 3.89 ppm showing through bond connectivities from Man 4H3.

proposed in [4] would perturb the intensity and multiplicity of the resonances at δ = 3.77 ppm in fig.4b. Moreover, using a variant of the basic COSY experiment to emphasize long-range couplings (see section 2), we have determined the resonance position of Man 4H5 to be ~3.5 ppm. The relevant cross-sections from the 2-D spectrum at ω_2 = 4.19 (Man 4H2) and ω_2 = 3.89 (Man 4H3) are shown in fig.5a,b. The assignments are clear from this representation. There are no large NOE's at δ ~3.5 ppm corresponding to NOE connectivities from Man 3H2 to Man 4H5 in either 1-D (fig.4b) or 2-D (fig.3) studies. The solution conformation of the Manα1–3Manβ1– unit in complex type structures is therefore correctly described by our previous 1-D NOE studies where we have quantified a series of interconnecting NOE's to determine a preferred solution conformation [1].

The crosspeaks from other resonances in fig.2 yield important information upon the solution conformation of other linkages in the molecule. For example, three crosspeaks along ω_2 = 4.44 ppm describe the through-space proton connectivities of Gal 6,6′ H1. Two of these, at 3.65 and 3.73 ppm, correspond to our previous assignments for Gal 6H3 and Gal 6H5 respectively, and are thus intra-residue NOE's. Furthermore, the multiplets at ~3.70 and 3.85 ppm correspond to inter-residue NOE's from Gal 6H1 to GlcNAc 5H4 (3.70 ppm) and possibly GlcNAc 5H6A(B) (3.85 ppm), within the α(1–3) antenna, with similar structures in the α(1–6) antenna. It is difficult to determine the conformations in each arm independently due to degeneracy of chemical shifts, but it is reasonable to assume that these are similar on grounds of symmetry. We emphasize that the absolute magnitudes of the NOE's in this case are not entirely suitable to define a unique solution conformation about the Galβ1–4GlcNAcβ1– linkage, due to their non-quantitative nature. However, the relative magnitudes of the inter-residue NOE's with respect to the intra-residue NOE between the uncoupled protons Gal6H1–Gal6H5, shows that a major conformer exists in solution, where Gal6H1 and GlcNAc 5H4 are in close proximity (<3 Å). Similarly, a crosspeak along ω_2 = 4.76 ppm (Man 3H1), at ω_1 ~3.7 ppm is proposed to arise from an NOE between Man 3H1 and GlcNAc 2H4, since all other crosspeaks at ω_2 = 4.76 ppm can be assigned to intra-residue NOE's (fig.2). Once again, the magnitude of this NOE relative to the intra-residue NOE between Man 3H1 and Man 3H5 argues for a dominant conformer, where Man 3H1 and GlcNAc 2H4 are within 3 Å of each other.

In contrast to the Man 4 residue in the α(1–3) arm, the Man 4′ residue in the α(1–6) arm does not generate large inter-residue NOE's to Man 3, nor can any observed NOE's be assigned to specific interactions with the core. However, small NOE's are observed from Man 4′ H1 to Man 3H5 and possibly Man 3H6 A(B) (not shown). However, these are insufficient to define the conformation of the Manα1–6Manβ1– unit due to the large number of conformers about the α(1–6) linkage. Their low intensity supports our previous conclusion that no single preferred conformer exists in solution about this linkage.

As in the α(1–3) arm, inter-residue NOE's are observed between GlcNAc 5′H1 and Man 4′H1 and between GlcNAc 5H1 and Man 4H1 (fig.2), suggesting that our previous 1-D NOE studies [1] adequately described the correct solution conformation of the GlcNAcβ1–2 Man unit in both antennae.

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